Remarks

Claims 25-39 are pending in the subject application. By this Amendment, Applicants have canceled claims 26-29 and amended claims 25, 33, and 35-39. Support for the amendments can be found throughout the subject specification, including the claim as filed. Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 25 and 30-39 are currently before the Examiner. Favorable consideration of the pending claims is respectfully requested.

Claims 25-39 are rejected under 35 USC §112, first paragraph, as nonenabled by the subject specification. The Examiner acknowledges that the claimed methods are enabled for using interferons and interferon chimeras, but asserts that the specification does not enable methods using "fragments of these molecules." Applicants respectfully assert that the claims are enabled by the subject specification. As the Examiner is aware, the enablement requirement of 35 USC §112 has two requirements: an application must teach an ordinarily skilled artisan "how to make" and "how to use" the claimed invention. Applicants respectfully assert that an ordinarily skilled artisan, having the benefit of the teachings of the subject application, can readily produce fragments of the various interferon proteins and test those fragments for biological activity (*e.g.*, the ability to downregulate IgE production). The level of skill of a person in the biotechnology arts is high. Methods for preparing fragments of a protein are well known in the art. In addition, methods for testing the fragments to determine if they have biological activity are well known in the art. Applicants respectfully submit that while some experimentation may be necessary to, it is not controlling on the issue of enablement where the experimentation is routine. *Ex parte Jackson*, 217 USPQ 804, 807 (Bd. Pat. App. & Int. 1982) ("The test [for undue experimentation] is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine . . .") (emphasis added). Preparation of protein fragments and the testing thereof for biological activity is routine in the art. Thus, Applicants respectfully assert that the subject specification teaches the ordinarily skilled artisan "how to make" and "how to use" the claimed invention. If the Examiner disagrees with any of the above statements, Applicants respectfully request that the Examiner provide evidence to demonstrate that the ordinarily skilled artisan would not be able to routinely produce and determine biological activity of fragments of an interferon molecule. In view of the above remarks,

reconsideration and withdrawal of the rejection under 35 USC §112, first paragraph, is respectfully requested.

Claims 25-39 are rejected under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner asserts that the subject specification does not provide written description of fragments of interferons that could be used in the claimed methods. Under this rejection, the Examiner asserts that "there is no description of the required structural and functional features of the such fragments. . . ." Applicants respectfully assert that there is adequate written description in the subject specification to convey to the ordinarily skilled artisan that they had possession of the claimed invention. Alpha, beta, tau, and gamma interferon proteins are well characterized in the art in regard to their structural and functional features. The primary amino acid sequence of alpha, beta, tau, and gamma interferon are well known in the art. As Applicants have stated in regard to the enablement rejection, methods for preparing and testing fragments from a full length protein are also well known in the art. Thus, it follows that the structure of any fragment of alpha, beta, tau, or gamma interferon is known in the art and every fragment can be tested for biological activity, without resort to undue experimentation, using techniques that are routine in the art. It is well settled in patent law that an application need not teach, and preferably omits, that which is well known or conventional in the art. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81 (Fed. Cir. 1986). Thus, there can be sufficient written description of an invention even if all the species of the genus are not explicitly disclosed in the specification. Accordingly, Applicants respectfully assert that the subject specification does provide written description for fragments of an interferon molecule as recited in the claimed methods. Reconsideration and withdrawal of the rejection under 35 USC §112, first paragraph, is respectfully requested.

Claims 26-32 and 36 are rejected under 35 USC §112, second paragraph, as indefinite. Applicants gratefully acknowledge the Examiner's observation that interferon gamma is not a type I interferon. By this Amendment, Applicants have amended the claims to delete reference to interferon gamma. In regard to the rejection of claim 36, Applicants respectfully assert that the claim is definite and that the ordinarily skilled artisan can ascertain the metes and bounds of the claimed

invention. However, in order to expedite prosecution of the subject application to completion, Applicants have amended the claim to recite that the IgE-related condition is an allergic condition. Accordingly, reconsideration and withdrawal of the rejection under 35 USC §112, second paragraph, is respectfully requested.

Claims 25, 26, 34, 38, and 39 are rejected under 35 USC §102(b) as anticipated by Pene *et al.* (1988). In addition, claims 26, 27, 33-37, and 39 are rejected under 35 USC §102(b) as anticipated by Gruschwitz *et al.* (1993). Finally, claims 25, 26, and 33-39 are rejected under 35 USC §102(b) as anticipated by Kimata *et al.* (1995). Applicants respectfully assert that the cited references do not anticipate the claimed invention. However, by this Amendment, Applicants have amended the claims to delete reference to interferon alpha and gamma. Accordingly, reconsideration and withdrawal of the rejections under 35 USC §102(b) is respectfully requested.

Claims 27-32 are rejected under 35 USC §103(a) as obvious over Pene *et al.* (1988), Gruschwitz *et al.* (1993), or Kimata *et al.* (1995), and further in view of Johnson *et al.* (WO 97/39127). The Examiner asserts that the cited references teach the use of interferon alpha to downregulate IgE production. While acknowledging that the primary references do not teach downregulation of IgE production by interferon tau, the Examiner asserts that it would have been obvious to substitute interferon tau for interferon alpha in view of the Johnson *et al.* reference which, according to the Examiner, teaches that interferon alpha and interferon tau bind to the type I receptor and have similar biological activities. The Johnson *et al.* reference is also cited as teaching interferon chimeras. Applicants respectfully traverse this grounds of rejection.

Applicants respectfully assert that the claimed invention is not obvious over the cited references, regardless of whether the references are taken alone or in combination. Although interferon alpha and interferon tau share some activities (for example, anti-viral activity), there are also numerous differences between interferon tau and the other type I interferons. These differences include the fact that interferon tau does not exhibit the level of toxicity exhibited by the other type I interferons, such as interferon alpha. Most notable among the differences between interferon tau and the other type I interferons is that interferon tau recognizes the type I interferon receptor complex differently than the other type I interferons. Experimental evidence suggests this differential recognition of the type I interferon receptor accounts for some of the differences and functions

observed between interferon tau and the other type I interferons. A publication by Subramaniam *et al.* (1995) (a copy of which is enclosed with this Amendment for the Examiner's convenience) reports the differential recognition of type I interferon receptor by interferon tau and interferon alpha. Experiments suggest that there are differences in the interaction at the amino termini of interferon tau and interferon alpha with the type I receptor complex. Thus, while interferon tau and other type I interferons share certain biological activities, Applicants respectfully assert that the ordinarily skilled artisan cannot predictably extrapolate activities associated with interferon alpha (or interferon beta or gamma) to interferon tau, in view of the significant differences between interferon tau and the other interferons.

As the Examiner is aware, it is well established in patent law that in order to support a *prima facie* case of obviousness, a person of ordinary skill in the art must find both the suggestion of the claimed invention, and a reasonable expectation of success in making that invention, solely in light of the teachings of the prior art. *In re Dow Chemical Co.*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). Applicants respectfully assert that the cited references do not teach or suggest the claimed invention. In addition, the requirement that there be a "reasonable expectation of success" in obtaining Applicants' claimed invention cannot be ignored in making an obviousness determination. Even assuming, *arguendo*, that the cited references suggested Applicants' claimed invention, they do not provide the requisite "reasonable expectation of success." In view of the fact that there are numerous differences between interferon tau and the other type I interferons, including, for example, their differential recognition of the type I interferon receptor complex, Applicants respectfully assert that the ordinarily skilled artisan would not have a reasonable expectation that interferon tau could be used to suppress or inhibit IgE production. Accordingly, reconsideration and withdrawal of the rejection under 35 USC §103(a) is respectfully requested.

It should be understood that these amendments have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

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DRP/sl
Attachments: Marked-Up Version of Amended Claims; publication by Subramaniam *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:12270-12274.

Marked-Up Version of Amended Claims

Claim 25 (amended):

25. A method for suppressing or inhibiting IgE production, said method comprising administering an effective amount of [a type I] interferon tau or a chimeric interferon, wherein said chimeric interferon comprises a mammalian interferon tau amino terminus and a human type I interferon carboxy terminus other than interferon tau, or a biologically active fragment [thereof] of said interferon tau or said chimeric interferon.

Claim 30 (amended):

30. The method according to claim [29] 25, wherein said mammalian interferon tau amino terminus is from a mammal selected from the group consisting of primate, ovine, and bovine.

Claim 31 (amended):

31. The method according to claim [29] 25, wherein said chimeric interferon comprises amino acid residues from about amino acid residue 1 to about amino acid residue 27 of ovine interferon tau and amino acid residues from about amino acid residue 28 to about amino acid residue 166 of human interferon alpha.

Claim 33 (amended):

33. The method according to claim 25, wherein said [type I] interferon tau or said chimeric interferon is administered to a person or animal in need of suppression or inhibition of IgE production.

Claim 35 (amended):

35. The method according to claim 33, wherein said [type I] interferon tau or said chimeric interferon is administered by routes selected from the group consisting of oral administration, parenteral administration, subcutaneous administration and intravenous administration.

Claim 36 (amended):

36. The method according to claim 35, wherein said person or animal is afflicted with, or predisposed to, an IgE-related condition, wherein said condition is an allergic condition.

Claim 37 (amended):

37. The method according to claim 36, wherein said [IgE-related condition is an] allergic condition is selected from the group consisting of allergic rhinitis, atopic dermatitis, bronchial asthma and food allergy.

Claim 38 (amended):

38. The method according to claim 25, wherein said [type I] interferon tau or said chimeric interferon is administered *in vitro*.

Claim 39 (amended):

39. The method according to claim 25, wherein said [type I] interferon tau or said chimeric interferon is formulated in a pharmaceutically acceptable carrier or diluent.

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Biochemistry

Differential recognition of the type I interferon receptor by interferons τ and α is responsible for their disparate cytotoxicities

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Communicated by George K. Davis, Gainesville, FL, September 6, 1995

ABSTRACT Interferon τ (IFNτ), originally identified as a pregnancy recognition hormone, is a type I interferon that is related to the various IFNα species (IFNαs). Ovine IFNτ has antiviral activity similar to that of human IFNαA on the Madin–Darby bovine kidney (MDBK) cell line and is equally effective in inhibiting cell proliferation. In this study, IFNτ was found to differ from IFNαA in that it was >30-fold less toxic to MDBK cells at high concentrations. Excess IFNτ did not block the cytotoxicity of IFNαA on MDBK cells, suggesting that these two type I IFNs recognize the type I IFN receptor differently on these cells. In direct binding studies, ^{125}I-IFNτ had a K_d of 3.90×10^{-10} M for receptor on MDBK cells, whereas that of ^{125}I-IFNαA was 4.45×10^{-11} M. Consistent with the higher binding affinity, IFNαA was severalfold more effective than IFNτ in competitive binding against ^{125}I-IFNτ to receptor on MDBK cells. Paradoxically, the two IFNs had similar specific antiviral activities on MDBK cells. However, maximal IFN antiviral activity required only fractional occupancy of receptors, whereas toxicity was associated with maximal receptor occupancy. Hence, IFNαA, with the higher binding affinity, was more toxic than IFNτ. The IFNs were similar in inducing the specific phosphorylation of the type I receptor-associated tyrosine kinase Tyk2, and the transcription factors Stat1α and Stat2, suggesting that phosphorylation of these signal transduction proteins is not involved in the cellular toxicity associated with type I IFNs. Experiments using synthetic peptides suggest that differences in the interaction at the N termini of IFNτ and IFNα with the type I receptor complex contribute significantly to differences in high-affinity equilibrium binding of these molecules. It is postulated that such a differential recognition of the receptor is responsible for the similar antiviral but different cytotoxic effects of these IFNs. Moreover, these data imply that receptors are "spare" with respect to certain biological properties, and we speculate that IFNs may induce a concentration-dependent selective association of receptor subunits.

Interferon τ (IFNτ), originally described as the major protein secreted by sheep conceptus, is functionally and structurally similar to the type I IFNs. Several IFNτ genes have been identified in sheep and cattle (1) and more recently in humans (2). Structurally, the amino acid sequence of IFNτ shows 30–70% homology to the type I interferons from various species (1). IFNτ functionally overlaps with IFNα. For example, ovine IFNτ is as potent an antiviral and antiproliferative agent as IFNα (3, 4) and is equally effective in induction of (2′–5′)oligoadenylate synthetase activity (5). However, unlike IFNα, IFNτ was found to be less cytotoxic at comparatively high doses *in vitro* (4). This is particularly significant since the type I IFNs are potential therapeutic agents for the treatment of various diseases that include cancers, autoimmune disorders, and viral diseases such as AIDS. However, toxic effects to cells and individuals often limit the dosage of IFN that can be used in therapy. The mechanism(s) by which IFNτ possesses potent antiviral and antiproliferative activities similar to those of IFNα while being far less toxic is unknown. We have begun an investigation of the mechanism for the lack of toxicity of IFNτ and show here that the cytotoxicity of the type I IFNs is dissociable from other properties such as antiviral activity.

The type I IFN receptor is a multisubunit protein complex (6, 7). It is conceivable, therefore, that the differential recognition of the receptor by different type I IFNs is the basis for overlapping but distinct biological activities of these cytokines. We tested the ability of ovine IFNτ to block the cytotoxicity of human IFNαA on Madin–Darby bovine kidney (MDBK) cells. Further, the IFNs were compared in binding competition experiments as well as at the level of signal transduction. The data suggest that IFNαA has higher avidity for receptor on MDBK cells than does ovine IFNτ and that this increased binding may be directly related to the differential toxicities but similar antiviral and antiproliferative properties of the type I IFNs.

MATERIALS AND METHODS

Materials. Purified recombinant human IFNαA (2×10^8 units/mg) was obtained from Biosource International (Camarillo, CA). Recombinant ovine IFNτ (0.8×10^8 units/mg) was expressed in *Pichia pastoris* and purified from the culture medium by ion-exchange and hydroxyapatite chromatography to homogeneity as determined by electrophoresis followed by silver staining. Antisera to peptides derived from the N terminus, IFNτ-(1–37), and C terminus, IFNτ-(139–172), of IFNτ have been described (8). Affinity-purified rabbit anti-peptide antibodies specific to Tyk2, Stat1α, and Stat2 were purchased from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Western blots were developed with an enhanced chemiluminescence (ECL) detection kit (Amersham).

Cell Lines and Cell Culture. The bovine kidney cell line MDBK and the human Burkitt lymphoma cell line Daudi were obtained from the American Type Culture Collection. The Daudi cells were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum and antibiotics. MDBK cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% horse serum and antibiotics.

Protein Assay. Protein concentration was determined with the bicinchoninic acid assay kit (Pierce) according to the manufacturer's instructions.

Antiviral Assay. Antiviral activity was quantitated by a cytopathic-effect-inhibition assay using vesicular stomatitis

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Abbreviations: IFN, interferon; MDBK, Madin–Darby bovine kidney.
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virus (9). Antiviral activity was normalized by using a value of 2×10^8 units/mg for IFNαA.

Cytotoxicity Assays. MDBK cells grown to confluence in 96-well plates were treated with appropriate concentrations of IFNs, in triplicate, in 100 μl of EMEM supplemented with 2% newborn calf serum. Control cells were treated with medium alone. Cells were incubated at 37°C until significant cell death was evident by microscopic examination. Cells were then stained with crystal violet, the plates were washed and air-dried, and the dye was extracted with 2-methoxyethanol (methyl cellosolve). Absorbance of the eluted dye was measured at 570 nm.

Labeling of IFNαA and IFNτ. IFNαA and IFNτ were labeled with the Bolton–Hunter reagent (mono [^{125}I]iodo derivative, ≈2000 Ci/mmol, Amersham; 1 Ci = 37 GBq) as described (10). Specific activity of labeled proteins was 40–70 μCi/μg. The labeled IFNs retained complete antiviral activity on MDBK cells which was unchanged for at least 4 weeks at 4°C.

For studies using anti-peptide antibodies, IFNα and IFNτ were biotinylated with the Immunopure NHS-LC-biotinylation kit (Pierce) according to the manufacturer's instructions.

Binding of ^{125}I-IFNαA and ^{125}I-IFNτ to MDBK Cells. Binding assays using MDBK cells were performed as described for IFNα (11). Confluent monolayers of MDBK cells in six-well plates precooled to 4°C were incubated with the appropriate concentrations of labeled or unlabeled IFNs in 2 ml of complete growth medium at 4°C for 4 hr for ^{125}I-IFNαA or overnight (≥17 hr) for ^{125}I-IFNτ to allow for binding to attain equilibrium. Saturation binding data were analyzed with the EBDA program (12).

For studies using anti-peptide antibodies, confluent monolayers of MDBK cells in 96-well plates were incubated with biotinylated IFNα or IFNτ at 0.3 μM in phosphate-buffered saline (PBS) containing 5% fetal bovine serum and a 1:30 dilution of the appropriate antiserum for 3 hr at room temperature. After washing with phosphate-buffered saline containing 5% fetal bovine serum, plates were developed with an ExtrAvidin-alkaline phosphate conjugate using *p*-nitrophenyl phosphate as substrate (Sigma).

Immunoprecipitation and Immunoblotting. Daudi cells in RPMI 1640 medium ($4–5 \times 10^7$ cells per 1-ml sample) stimulated with IFNαA or IFNτ at 5000 units/ml were lysed at 4°C for 20 min in 500 μl of ice-cold lysis buffer consisting of 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 mM β-glycerol phosphate, 2 mM Na_3VO_4, 0.05 mM *p*-nitrophenyl-*p'*-guanidinobenzoate (from a stock solution in dimethylformamide), leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), benzamidine (5 μg/ml), 1 mM phenylmethanesulfonyl fluoride, 10% (vol/vol) glycerol, and 1% (vol/vol) Nonidet P-40. After immunoprecipitation of extracts (500 μl) with 1 μg of anti-Tyk2, or a mixture of 1 μg of anti-Stat1α and 1 μg of anti-Stat2, and immunoblotting, tyrosine phosphorylation of Tyk2, Stat1α, and Stat2 was detected with anti-phosphotyrosine (4G10) antibody and ECL. Blots were then stripped and reprobed for the appropriate proteins with the corresponding antibodies.

RESULTS

We first determined the relative cytotoxicities of recombinant human IFNαA and recombinant ovine IFNτ at various concentrations on MDBK cells (Fig. 1). IFNτ was at least 30-fold less toxic to MDBK cells. The concentration causing 50% cell death was about 2500 units/ml for IFNα in comparison to about 85,000 units/ml for IFNτ. Thus, IFNτ and IFNα differ markedly in their cytotoxic effects.

The potential of IFNτ at subtoxic concentrations to act as a competitive antagonist to the cytotoxic effect of IFNα was next investigated. IFNτ at a 10-fold higher (but subtoxic) concentration than IFNαA did not block the toxicity of IFNαA on MDBK cells (Fig. 2*A*). Moreover, addition of IFNτ at a 5-fold higher concentration 1 hr prior to the addition of IFNαA to the cells (Fig. 2*B*) also did not block the toxicity of IFNαA. Thus, IFNτ is a poor antagonist to the cytotoxic effect of IFNαA. This was surprising in view of the established structural and functional homology of these two type I interferons (13) and their similar specific antiviral activities on MDBK cells (3). The inability of IFNτ to block the cytotoxicity of IFNαA suggests that these IFNs bind differently to the type I receptor complex, perhaps initiating different signaling events.

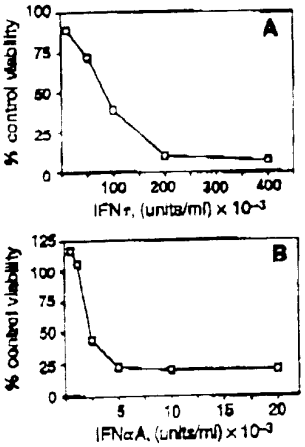

FIG. 1. Cytotoxicity profiles of IFNαA and IFNτ on MDBK cells. Confluent monolayers of cells in 96-well plates were treated with either IFNτ (*A*) or IFNαA (*B*) at the indicated concentrations (antiviral units). Control wells were left untreated.

Consequently, experiments to follow the direct binding of IFNτ to MDBK cells and the competitive binding of IFNα were undertaken with ^{125}I-labeled IFNτ and IFNαA. ^{125}I-IFNτ bound to MDBK cells with high specificity (Fig. 3*A*). Scatchard analysis of the binding (*Inset* in Fig. 3A) revealed an apparent K_d of 3.90×10^{-10} M. The K_d value is within the range (10^{-11} to 10^{-9} M) for the binding of the various IFNαs to a variety of cell types (14, 15) and is similar to the value for binding of

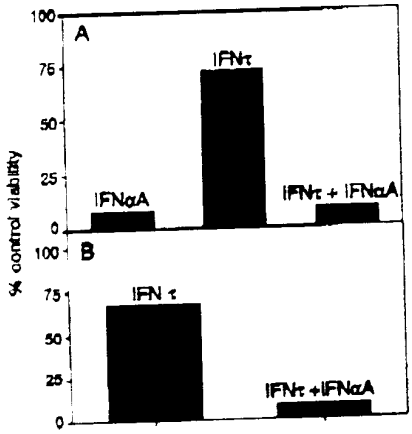

FIG. 2. Effect of IFNτ on the cytotoxicity of IFNαA on MDBK cells. (*A*) IFNτ (50,000 units/ml) and/or IFNαA (5000 units/ml) were added to cells. (*B*) Cells were treated for 1 hr at 37°C with IFNτ (25,000 units/ml) before the addition of IFNαA (5000 units/ml) without removal of IFNτ.

recombinant bovine IFNαD (3.5×10^{-10} M) and recombinant bovine IFNτ (3.7×10^{-10} M) to bovine endometrial membranes (16). However, Scatchard analysis of the binding of ^{125}I-IFNαA to MDBK cells (Fig. 3*B*) yielded an apparent K_d of 4.45×10^{-11} M for IFNαA, similar to that (6.0×10^{-11} M) previously reported (11). The total receptor concentration for ^{125}I-IFNαA (4.62 pM) was very similar to that for ^{125}I-IFNτ (4.22 pM) from the Scatchard plots. Thus, IFNαA has close to a 10-fold lower K_d for receptor on MDBK cells. This large difference in binding affinities may be responsible for the inability of IFNτ to "compete" for receptor and block the toxicity of IFNαA.

This difference in the affinities of IFNαA and IFNτ for receptor on MDBK cells was clearly reflected in binding competition experiments (Fig. 4). IFNαA was a potent competitor of ^{125}I-IFNτ binding to MDBK cells (Fig. 4*A*). In fact, IFNαA was 40-fold more effective than IFNτ itself in inhibiting the binding of ^{125}I-IFNτ at the 50% level. Similar results were obtained when recombinant human IFNαD was used as the competitor (data not shown). Cross-competition studies using ^{125}I-IFNαA (Fig. 4*B*) again showed that IFNαA was >40-fold more effective than IFNτ in displacing ^{125}I-IFNαA at the 50% level. Clearly, IFNαA has a much higher affinity than IFNτ for the receptors on MDBK cells, which is consistent with the 10-fold lower K_d. Moreover, the results of the competition experiments provide an explanation for the inability of excess (5:1 ratio) IFNτ to block the cytotoxic effects of IFNαA on MDBK cells. The higher receptor affinity of IFNαA suggests differential receptor recognition by IFNτ and IFNαA, and explains the inability of IFNτ to block the toxicity of IFNαA.

Using synthetic overlapping peptides representing the complete sequence of ovine IFNτ, we have shown previously that a peptide encompassing the N-terminal 37 amino acids [IFNτ-(1–37)] specifically blocked the antiviral activity of IFNτ, but not that of IFNα, on MDBK cells (8). In contrast, peptides corresponding to the C-terminal amino acids, 139–172, blocked the antiviral activity of both IFNα and IFNτ. These data suggested that the N-terminal region of IFNτ bound differently to the receptor on MDBK cells. Related to this, we next determined the ability of rabbit antisera to these peptides to block the binding of IFNα and IFNτ to MDBK cells. Antiserum to IFNτ-(1–37) specifically blocked the binding of IFNτ, but not that of IFNα, to MDBK cells. By contrast, antiserum to the C-terminal peptide IFNτ-(139–172) blocked binding of both IFNτ and IFNα (Fig. 5). These findings are consistent with the hypothesis that IFNτ-(1–37) contains an epitope that is unique to IFNτ and is crucial for both binding and biological activity of IFNτ on MDBK cells. Furthermore, such a differential interaction at the N termini of IFNτ and IFNα could account in part for the differential competition at the receptor on MDBK cells.

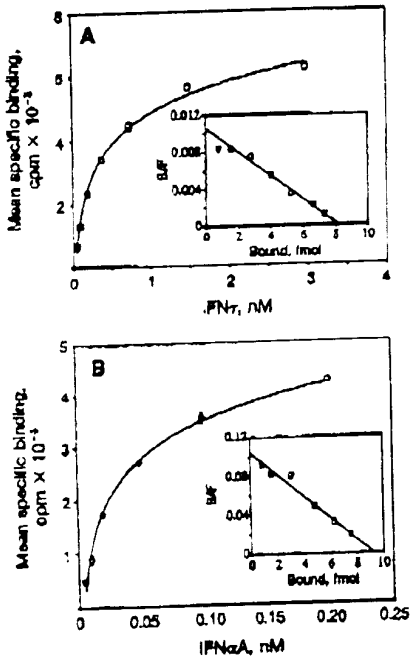

FIG. 3. Binding of ^{125}I-IFNτ (*A*) and ^{125}I-IFNαA (*B*) to MDBK cells. Specific binding was calculated by subtracting nonspecific binding determined at each concentration in the presence of a 100-fold excess of the corresponding unlabeled IFN. Nonspecific binding was <20% for IFNτ and <7% for IFNαA. Values are plotted as mean ± SE. (*Insets*) Scatchard plots of binding data. B, bound; F, free.

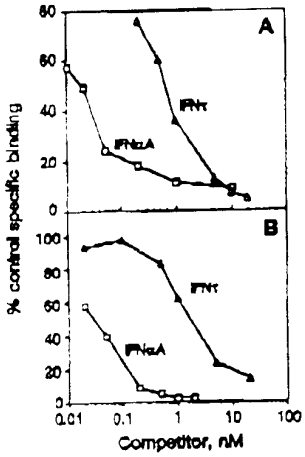

FIG. 4. Competitive binding of IFNτ and IFNαA to MDBK cells. Confluent monolayers of MDBK cells in six-well plates were incubated, in triplicate, with the indicated concentrations of unlabeled IFNαA or IFNτ and 0.2 nM ^{125}I-IFNτ (*A*) or 0.02 nM ^{125}I-IFNαA (*B*). Values are presented as percent specific binding of control determined in the absence of competitor. A 100% value represents a mean specific binding of 1673 ± 51 cpm (mean ± SE) for ^{125}I-IFNτ and 1255.7 ± 16.3 cpm (mean ± SE) for ^{125}I-IFNαA. Nonspecific binding was determined in the presence of 200 nM unlabeled IFNτ for ^{125}I-IFNτ (11% nonspecific binding) and 20 nM unlabeled IFNαA for ^{125}I-IFNαA (5% nonspecific binding) and was subtracted from total binding.

The antiviral activities of IFNτ and IFNαA are presented in Fig. 6 as concentration–effect curves. Cytotoxicity is associated with saturation binding to the receptor, whereas antiviral activity involves fractional occupancy of the receptors. Thus, toxicity is associated with the K_d. IFNαA binds to MDBK cells with 10-fold greater affinity than IFNτ and is toxic at much lower concentrations. The specific antiviral activities of the two IFNs are similar, 2×10^8 units/mg of protein for IFNαA and 0.8×10^8 units/mg for IFNτ, which is consistent with the induction of maximal antiviral activity with only a low fractional occupancy of receptors.

Binding of IFNs to receptors is translated within the cell by signal transduction machinery involving a set of tyrosine kinases and transcription factors that are activated by phosphorylation. Thus, we investigated whether the differences in binding of IFNα and IFNτ are manifest in changes in activation of the type I receptor-associated tyrosine kinase Tyk2 and the transcription factors Stat1α and Stat2. The phosphorylation of

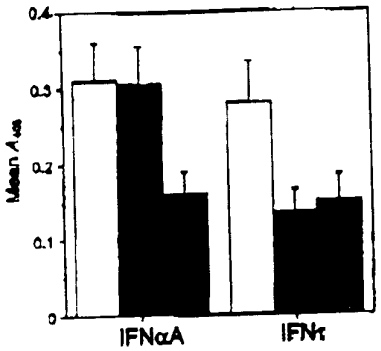

FIG. 5. Inhibition of binding of IFNτ and IFNαA to MDBK cells by antisera raised against the N terminus (solid bar) and C terminus (stippled bar) of IFNτ. Data are presented as mean absorbance ± SD. Control samples were treated with preimmune serum (open bars).

each of these proteins induced upon stimulation of cells by IFNα and IFNτ was followed by immunoprecipitation with specific antiserum and Western blotting with anti-phosphotyrosine antibody. IFNτ (Fig. 7A, lane 3) was as effective as IFNα (lane 2) in the phosphorylation of Tyk2. No differences were seen in the levels of Tyk2 protein in unstimulated cells and cells stimulated with the IFNs (Fig. 7B).

IFNτ (Fig. 8A, lane 3) and IFNα (lane 2) induced comparable levels of phosphorylation of both Stat1α and Stat2. Again, no differences were seen in the protein levels of the Stat1α and Stat2 immunoprecipitated from stimulated or unstimulated cells (Fig. 8B). Thus, IFNτ is similar to IFNα in activating these signal transduction proteins associated with

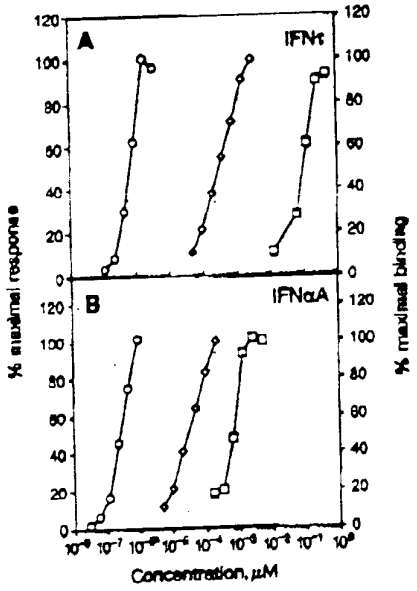

FIG. 6. Dose–response/occupancy curves for IFNτ (A) and IFNαA (B) on MDBK cells. Percent maximal antiviral activity (○), percent maximal cytotoxicity (□), and percent maximal binding (◇) as determined on MDBK cells are plotted as functions of concentration. Data from cytotoxicity dose–response curves and saturation binding curves were replotted as percent maximal values. Concentrations were determined from antiviral units, using a specific activity of 2.0×10^8 units/mg for IFNαA and 0.8×10^8 units/mg for IFNτ on MDBK cells.

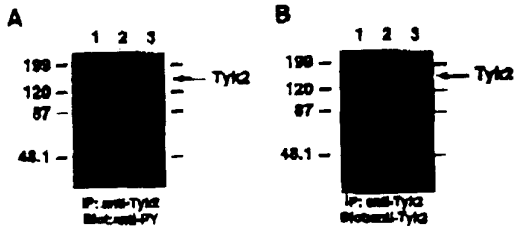

FIG. 7. Tyk2 phosphorylation induced by IFNτ and IFNαA. Daudi cells were stimulated with IFNαA (lane 2) or IFNτ (lane 3) at 5000 units/ml or were left untreated (lane 1) for 4 min at 37°C. After immunoprecipitation (IP) with antibodies to Tyk2 (anti-Tyk2) and immunoblotting, the blot was probed with monoclonal anti-phosphotyrosine (anti-PY) antibody (A), stripped of anti-PY, and probed with antibodies to the Tyk2 protein (B). Molecular size (kDa) markers are at left.

the type I receptor, in keeping with its structural and functional similarities with the IFNαs.

DISCUSSION

IFNτ, identified originally as a major protein in the reproductive cycle in sheep and cows, is now recognized as a type I IFN whose biological properties closely parallel those of the IFNαs. Ovine IFNτ is as potent as IFNα in antiviral and anticellular activities, but, unlike IFNα, it is relatively nontoxic to cells and does not suppress bone marrow at high doses (17). We have previously shown (8), using peptide antagonists, that the C terminus of ovine IFNτ effectively antagonized the properties of both IFNα and IFNτ whereas the N terminus of IFNτ antagonized only the properties of IFNτ.

Consistent with these peptide antagonist studies, the present study shows that high concentrations (up to a 10-fold excess) of ovine IFNτ failed to compete for receptor and block the toxic effects of human IFNαA on MDBK cells. A comparison of the relative antiviral, cytotoxic, receptor binding, and receptor competition properties of ovine IFNτ and human IFNαA provides insight at the level of ligand–receptor interactions. IFNτ and IFNαA possess similar specific antiviral activities, as previously shown (3). However, IFNαA has an ~10-fold lower K_d for receptor than does IFNτ and, hence, a higher binding affinity for receptor. Moreover, IFNαA is severalfold more effective than IFNτ in binding competition assays using either ^{125}I-IFNτ or ^{125}I-IFNαA. Since the numbers of binding sites per cell for IFNτ and IFNαA are very similar, and IFNτ competes with IFNαA binding, it appears that IFNαA and IFNτ recognize the same receptor complex. A comparison of dose–response/occupancy curves for the cyto-

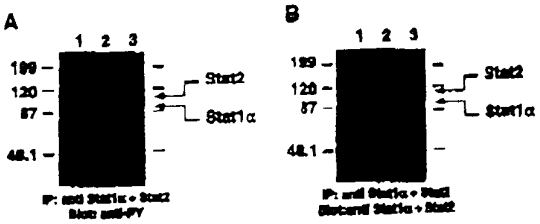

FIG. 8. Stat1α and Stat2 phosphorylation induced by IFNαA and IFNτ. Daudi cells were stimulated with IFNαA (lane 2) or IFNτ (lane 3) at 5000 units/ml or were left untreated (lane 1) for 10 min at 37°C. After immunoprecipitation (IP) with a mixture of antibodies to Stat1α and Stat2 (anti Stat1α + Stat2) and immunoblotting, the blot was probed with anti-phosphotyrosine (anti-PY) antibody (A), stripped of anti-PY, and probed with a mixture of antibodies to Stat1α and Stat2 (B).

toxicities and antiviral activities shows that cytotoxicity is associated with maximal receptor occupancy and hence binding affinities; IFNαA has a greater binding affinity and thus possesses much greater toxicity. On the other hand, antiviral activity is maximal at concentrations that result in only a very small fractional occupancy of receptors and is not represented by equilibrium binding data. This is consistent with the concept that the receptors on MDBK cells are "spare" in number with respect to antiviral activity. "Spareness" of receptors in a multisubunit complex receptor could be envisaged as arising from the differential interaction of ligand with a subset of receptor subunits or from differences in the levels of expression of the subunits. Selective interactions may arise from differential affinities of the subunits for ligand. Such phenomena can be recognized, for example, in the case of some interleukin receptors (18). Also, if the concentration of a cellular effector molecule is limiting with respect to a given response, receptors would appear spare in number, since maximal response would occur without maximal occupancy.

In this regard, we have shown in this report that ovine IFNτ, like IFNαA, can induce the very rapid phosphorylation of the type I receptor-associated kinase Tyk2 and the transcription factors Stat1α and Stat2. Given the time scales of the stimulation by IFNτ and IFNαA, it appears that only a small fraction of the receptors need to be occupied in order to induce the phosphorylation of Tyk2, Stat1α, and Stat2. Taken together, these data suggest that phosphorylation of these signal transduction proteins may not be sufficient to induce the cellular toxicity associated with the type I IFNs.

The higher binding affinity of IFNαA for receptor and the differential competition properties of IFNτ and IFNαA also suggest that the IFNs recognize the receptor differently. Studies from our laboratory with synthetic peptide antagonists showed that the C-terminal peptide IFNτ-(139–172) was competitive against the activities of both IFNαA and IFNτ, whereas the N-terminal peptide IFNτ-(1–37) was effective at a 5- to 10-fold higher concentration only against IFNτ activity (8, 19). Using antisera raised against IFNτ-(139–172) and IFNτ-(1–37), we have shown that antiserum to IFNτ-(1–37) blocks only the binding of IFNτ, whereas antiserum to IFNτ-(139–172) blocks the binding of both IFNα and IFNτ. These data suggest that the N-terminal portions of IFNτ and IFNαA represent significant determinants of high-affinity binding and that differences in high-affinity equilibrium binding between IFNαA and IFNτ may in good part be due to differences in receptor interactions at the N termini of these molecules. Consequently, the N termini of these molecules also appear to be significant determinants of the cytotoxic effects of the IFNs.

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1. Roberts, R. M., Cross, J. C. & Leaman, D. W. (1992) *Endocr. Rev.* **13**, 432–452.
2. Whaley, A. E., Reddy Meka, C. S., Harbison, L. A., Hunt, J. S. & Imakawa, K. (1994) *J. Biol. Chem.* **269**, 10864–10868.
3. Pontzer, C. H., Torres, B. A., Vallet, J. L., Bazer, F. W. & Johnson, H. M. (1988) *Biochem. Biophys. Res. Commun.*, **152**, 801–807.
4. Pontzer, C. H., Bazer, F. W. & Johnson, H. M. (1991) *Cancer Res.* **51**, 5304–5307.
5. Mirando, M. A., Short, E. C., Jr., Geisert, R. D., Vallet, J. L. & Bazer, F. W. (1991) *J. Reprod. Fertil.* **93**, 599–607.
6. Uze, G., Lutfalla, G. & Mogensen, K. E. (1995) *J. Interferon Cytokine Res.* **15**, 3–26.
7. Cleary, C. M., Donnelly, R. J., Soh, J., Mariano, T. M. & Pestka, S. (1994) *J. Biol. Chem.* **269**, 18747–18749.
8. Pontzer, C. H., Ott, T. L., Bazer, F. W. & Johnson, H. M. (1994) *J. Interferon Res.* **14**, 133–141.
9. Armstrong J. P. (1981) *Methods Enzymol.* **78**, 381–387.
10. Langer, J. A. & Pestka, S. (1986) *Methods Enzymol.* **119**, 305–311.
11. Zoon, K. C., Zur Nedden, D. & Arnheiter, H. (1986) *Methods Enzymol.* **119**, 312–315.
12. McPherson, G. A. (1985) *J. Pharmacol. Methods* **14**, 213–228.
13. Jarpe, M. A., Johnson, H. M., Bazer, F. W., Ott, T. L., Curto, E. V., Rama Krishna, N. & Pontzer, C. H. (1994) *Protein Engin.* **7**, 863–867.
14. Rubinstein, M. & Orchansky, P. (1986) *CRC Crit. Rev. Biochem.* **21**, 249–275.
15. Aguet, M. & Mogensen, K. E. (1983) in *Interferon*, ed. Oresser, I. (Academic, New York), Vol. 5, pp. 1–22.
16. Li, J. & Roberts, R. M. (1994) *J. Biol. Chem.* **269**, 13544–13550.
17. Soos, J. M., Subramaniam, P. S., Hobeika, A. C., Schiffenbauer, J. & Johnson, H. M. (1995) *J. Immunol.* **155**, 2747–2753.
18. Lopez, A. F., Elliot, M. J., Woodcock, J. & Vadas, M. A. (1992) *Immunol. Today* **13**, 495–500.
19. Pontzer, C. H., Ott, T. L., Bazer, F. W. & Johnson, H. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5945–5949.